BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

4453348 10 5/12

STIC-ILL

From: White, Everett
Sent: Monday, May 12, 2003 10:41 AM
To: STIC-ILL
Subject: Reference Ordering

Template

ILL Ordering Information:

10534012

Art Unit or Location: 1623

Telephone Number: 308-4621

Application Number or Other Order Identifier: 09/118,730

The following references are requested: The information for the references disclosed below were obtained from U.S. Patent No. 4,808,576. The name of the journal appears to be missing from the Peyron et al reference. However, a search of the reference from the information disclosed below would be appreciated.

Preliminary Clinical Assessment of NaHyaluronate Injection Into Human Arthritic Joints, by Peyron et al, Vol. 22, No. 8, pp. 731-736, Oct., 1974.

Decreased Granulation Tissue Reaction After Installment of Hyaluronic Acid, by Rydell, pp. 307-311, Vol. 41, of Acta Orthop Scandinav.

Clinical Orthopaedics, pp. 25-32, Oct., 1971, No. 80, by Rydell et al, "Effect of Intra-Articular Injection of Hyaluronic Acid on Clinical Symptoms".

Effect of Intra-articular Injection of Hyaluronic Acid on the Clinical Symptoms of Osteoarthritis and on Granulation Tissue Formation

NILS RYDELL, M.D. AND ENDRE A. BALAZS, M.D.

This paper was initiated by the electron microscopic studies of Balazs, Bloom and Swann[2] who found a 1–2 μ thick layer adherent to the articular cartilage surface and found that it may contain hyaluronic acid. This layer seems to be associated with the aging processes because it becomes thicker in older animals.

Enzymatic, electron microscope and chemical studies performed on fresh joint specimens from cows support the idea that this layer contains hyaluronic acid.[1] Hyaluronic acid may, therefore, be important not only as a constituent of synovial fluid, but also as a component of the cartilage surface. Such a layer of hyaluronic acid on the cartilage surface may protect the cartilage from wear and also act as a shock absorber protecting the cartilage cells from shockwaves. In the vitreous of the eye the hyaluronic acid gel seems to be an excellent shock absorber for the retina.[3]

Under dynamic conditions, hyaluronic acid solutions have a significant viscoelastic behavior. The biological importance of this was pointed out by Balazs and co-workers.[3, 6]

In normal joints the hyaluronic acid layer forms an elastic cushion between the joint surfaces and behaves as a good shock absorber.[3]

If for some reason the hyaluronic acid layer on the cartilage surface disappears, the cartilage cells may be damaged by impact on the joint surface and/or the superficial part of the cartilage will be submitted to mechanical wear.

If in arthritic joints this layer of hyaluronic acid on the cartilage surface could be restored by injections of highly concentrated hyaluronic acid solutions, the condition could improve and the progress of the disease might be arrested.

METHODS AND MATERIALS

The hyaluronic acid used in these experiments was prepared from human umbilical cords and rooster combs. The sterile preparations were obtained as previously described.[4]* These preparations have a protein content of 0.2–0.3 per cent and a molecular weight of 1–2 × 10^6. The intrinsic viscosity is in the range of 3,000 cc/g. The sodium salt of hyaluronic acid was dissolved in saline to a concentration of 3–8 mg/ml. Two to 3 milliliters of the solution (6–24 mg of hyaluronic acid) were used each time for joint injections.

From the Department of Connective Tissue Research, Boston Biomedical Research Institute, Boston, Massachusetts, and the Orthopedic Clinic, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts.

Supported in part by a grant from The John A. Hartford Foundation, New York, N. Y., by a PHS research grant (EY-00223) from the National Eye Institute, U. S. Public Health Service; and by a Special Fellowship (IF05TW1266-01) from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

* This hyaluronic acid preparation now is available from Biotrics, Inc., 24 Beck Road, Arlington, Massachusetts, 02174.

Experiments were carried out on both knee-joints of dogs and owl monkeys by creating a surgical wound in the weight-bearing area of the lateral femoral condyle. In one joint, hyaluronic acid was injected at the time of the operation and on every fourth day for 4 weeks. Nothing was injected into the other joint, as saline injections are known to be harmful to the joints. After 6–8 weeks the animals were sacrificed and the two sides compared.

Traumatic and degenerative arthritis in race horses is a frequent and disabling joint disorder for which corticosteroids are generally used. The intra-articular injections of suspensions of water-insoluble salts of corticosteroids offer only temporary improvement in the majority of cases, and prolonged use of the drug can cause considerable damage to the articular cartilage. The fact that traumatic arthritis is frequently observed in young animals which are heavily raced and that the changes usually take place in the carpal, tarsal, fetlock, and coffin joints, which are all significantly stressed during racing, suggests that these changes are caused by wear and tear, although such factors as nutrition and heredity are probably also of importance.[7, 8]

In the first series of treated horses presented here,[9] the hyaluronic acid was mixed with Depo-Medrol (6-α-methylprednisolone 21-acetate, Upjohn). In the control group, only Depo-Medrol was injected.

Further studies with hyaluronic acid injections alone have given results as good as those from cases treated with both hyaluronic acid and cortisone.

Twenty race horses (aged 3–10 years) with traumatic or degenerative arthritis were used. In most cases only one joint was involved, but in a few cases two diseased joints of the same animal were treated.

Six of the horses had been treated earlier with Depo-Medrol with no or only short-lasting effects; recurrence of symptoms occurred within 1–4 weeks. Almost all joints involved were swollen and had increased skin temperature compared to the joint of the opposite side. In a few cases, a considerable joint effusion under pressure was observed. In these cases the excess synovial fluid was removed from the joint. In all other cases, the volume of the synovial fluid removed was the same as the injected volume.

The treatment and evaluation of the results are described in 2 series. Twelve horses receiving cortisone and hyaluronic acid constitute Series I; 8 horses receiving cortisone alone constitute Series II. The selection of horses for Series I and II were randomly made. Each series is subdivided into 3 groups according to the animal's ability to use the joint while racing, walking, and standing:

Group I horses showed symptoms of functional disorder only during extreme stress on the joint; viz., as bearing out or in, during racing.

Group II horses showed symptoms of lameness, limping at any time when in motion and when the joint was used, but not while standing.

Group III horses showed symptoms when standing and refusing to place their full weight on the diseased joint under any conditions.

In all 3 groups the skin over the diseased joint was usually considerably warmer than normal.

The effect of treatment was evaluated on a functional basis, i.e., whether or not the horse could return to racing, how well the animal performed, and the duration of improvement. The results were defined as follows: Poor—the horses continued to be lame and unable to race, or the lameness reappeared after a short period of improvement (less than 4 weeks) and the horse was unable to race in spite of repeated injections; fair—the horse could race again, but did not perform as well as immediately prior to the onset of symptoms; good—the horse returned to racing a few days after treatment, performing as well as it did before the symptoms started for a period of at least 3 months (this 3-month period was arbitrarily selected because we did not have the opportunity to follow the 2 cases in this group for a longer period of time); excellent—the horse resumed racing, performing as well as or better than it had prior to the manifestation of the symptoms, and the improvement was maintained for more than 3 months.

In order to investigate the effect of the implantation of hyaluronic acid on the subcutaneous scar formation after surgical incision, 45 animals were each subjected to incisions 4 cm long, symmetrically on both sides of the body, reaching down to the muscular fascia. The fascia was traumatized by 10 strokes of a gauze pad. It was considered of great importance that exactly the same damage be performed on both sides of the same animal. Twenty-five rabbits, 10 owl monkeys, and 10 guinea pigs (a total of 45 animals) were used. On one side of each animal, hyaluronic acid was implanted before the wound was closed; on the other side, the control side, saline was injected in 2 cases. As the injection of saline alone may be harmful to the connective tissue, this procedure was avoided in most animals. The surgical procedure was identical on both sides. Three to 6

weeks after the operation, each animal was sacrificed and the subcutaneous part of the scar explored. The result was evaluated by comparing the reactions of both sides of the same animal. On each side the reaction was classified by 3 grades. Grade 1 classification was used when the adhesions formed were thin and sparse and easily broken; grade 2 indicated a more dense adhesion formation, but one which was easily disrupted on dissection; the reaction was classified as grade 3 if the adhesions formed a dense tissue and a knife was necessary for dissection.

In order to find out if hyaluronic acid could also be used in decreasing connective tissue reaction around foreign body implants, the following experiment was performed: An incision 3 cm long was made in the lower region of the thorax of each of 5 guinea pigs. The incision was placed on each side 2 cm from the midline of the dorsum, with its midpoint over the tip of the tenth rib. On each side 2 hollow polyethylene tubes, each 1 cm long, with a 10 mm outer diameter and a 6 mm inner diameter, were implanted. One of the tubes was placed under the muscle fascia and the other subcutaneously. On one side, hyaluronic acid was implanted around the tubes. On the other side saline solution was injected around the tubes. The fascia and skin were closed by silk sutures. Within the next few days, 2 of the animals showed a marked wound reaction on the saline-injected side and later the implanted tubes were rejected. The wounds then healed within a few days. The 3 animals which did not reject the tubes on the control side showed a marked reaction in the wound area, and a large mass of granulated tissue could be palpated. The tubes implanted in hyaluronic acid did not cause any inflammatory reaction, and in all animals the wound healed nicely within a few days. Six weeks after the operation, the animals were sacrificed, the subcutaneous wound area explored, and the capsule around the tubes was studied histologically.

In another experiment, the following procedure was followed: in 35 rabbits weighing between 2.5 and 4 kg the medial and anterior parts of their hindlegs were shaved and 1–2 days later they were operated on under sterile conditions using pentothal anesthesia. A skin incision 3.5 cm long was made over the long extensor tendon of the great toe. The tendon sheath was incised along its anterior attachment to the tibia, and the tendon was carefully released from the surrounding tissue. The incision of the tendon sheath was started 0.5 cm above the tip of the medial malleolus and extended 3.5 cm in the cranial direction. A standardized trauma was applied on the dorsal part of the tendon by rubbing it 20 times with the serrated area of a forceps. The ventral part was rubbed 30 times with a piece of dry gauze.

In each rabbit hyaluronic acid was implanted into the tendon sheath on one side and the tendon sheath was then closed by 2 silk sutures. On the other side, which was used as control, the sheath was directly closed after the application of trauma, again using 2 silk sutures. On both sides 4 silk sutures were used for skin closure. Physiologic saline solution was not injected into the tendon sheath of the control side since this treatment is known to produce adhesions. Three weeks after the operation the animals were sacrificed. The hind legs were amputated above the knee. A small skin incision over the medial malleolus was made and the tendon was divided 2 mm proximal to the ligament which keeps the tendon in the groove of the medial malleolus. Another small incision was made proximally below the knee, and the tendon was dissected free from the skin and divided 5 cm above the medial malleolus. At this point the tendon changes from a conical to a cylindrical shape. A silk suture was fastened to the proximal end of the separated part of the tendon, and this was freed from surrounding tissue. A spring balance was attached to the silk, the force necessary to pull the tendon out of its sheath was measured, and the recorded value was taken as a measure of adhesion formation.

RESULTS

In the 2 dogs and 6 owl monkeys the damaged knee joints, after hyaluronic acid treatment, were smoother and showed less reaction than the non-injected sides. This side also showed an increased amount of synovial fluid content as a sign of irritation.

Independent of our work, a similar study was carried out on rabbits, reporting improved healing of cartilage after implantation of hyaluronic acid.[5]

In all cases of race horses with traumatic arthritis a decrease in swelling was recorded after each injection. However, the swelling seemed to decrease faster after injection of hyaluronic acid mixed with cortisone than after injections of cortisone alone. The skin temperature decreased after injection of hyaluronic acid and cortisone; this was observed

TABLE 1. Summary of the Clinical Evaluation of Intra-articular Hyaluronic Acid-Cortisone Treatment and Cortisone Treatment

	Group	No. of Horses in Each Group	Evaluation			
			Poor	Fair	Good	Excellent
Hyaluronic acid and cortisone injection	I	4				4
	II	3				3
	III	5		1	1	3
	Total	12		1	1	10
Cortisone injection	I	4	3			1
	II	2	2			
	III	2	2			
	Total	8	7			1

less frequently after injections of cortisone alone.

Table 1 summarizes results of the treatment in both series. It is obvious that the cortisone treatment in combination with hyaluronic acid resulted in a more lasting and better improvement than when cortisone alone was used. Seven horses treated with hyaluronic acid and cortisone were able to return to racing within a short period of time and performed well without further treatment for the entire follow-up period. Three other horses required one additional injection in order to perform as well; results of this treatment were judged to be excellent. Two horses in this series showed about the same degree of improvement, but since they could be followed for only 3 months, the evaluation of the treatments was judged to be fair and good, respectively.

Six horses in Series 1 were treated with cortisone prior to the hyaluronic acid-cortisone injections. Since cortisone treatment alone did not improve the function of the diseased joint, the improvement observed in these cases after hyaluronic acid treatment is especially significant.

Cortisone treatment of 8 horses produced immediate improvement and, in most cases, joint function was so good that the horses were able to race well for short periods of time. In 7 cases, however, improvement was temporary. After repeated injections of cortisone the symptoms remained unchanged and, in many cases, rapid deterioration of the function of the joint was obvious after continued exercise.

One of the 45 animals used for studies of scar formation and wound healing died in connection with the operation, and 5 developed infection. These were excluded from this study. Of the 39 animals remaining for evaluation, 32 showed less connective tissue reaction with a smoother subcutaneous scar on the hyaluronic acid-treated side, 6 showed no difference between the two sides, and in one case the control side had the smoothest healing (Table 2).

Of the 10 owl monkeys, 2 were excluded due to infection. In 6 of the monkeys, the hyaluronic acid-treated side was classified as grade 1 and the control side as grade 2; in 2 cases, both sides were marked as grade 2. Four of the 25 rabbits were excluded, 1 due to death and 3 due to infection. Of the remaining 21 rabbits, 16 revealed less reaction on the hyaluronic acid-treated side (12 classified as grade 1; 4 as grade 2); on

TABLE 2. Subcutaneous Tissue Reaction to Surgical Skin Incision With and Without Hyaluronic Acid Implantation

Animals	*Number Evaluated*	*Less Reaction on Hyaluronic Acid Side*	*Less Reaction on Control Side*	*No Difference Between Hyaluronic Acid and Control Sides*
Owl monkey	8	6	0	2
Rabbit	21	16	1	4
Guinea pig	10	10	0	0
Total	39	32	1	6

the control side, all 16 were classified as grade 3. Four rabbits showed no difference between the two sides; 3 of them were classified as grade 1 and one as grade 2 on each side. One rabbit had smoother healing on the control side, marked as grade 1, while the hyaluronic acid-treated side was marked grade 2.

None of the 10 guinea pigs was excluded. All were classified as grade 1 on the hyaluronic acid-treated side and grade 2 on the control side.

Studies of foreign-body reaction with and without hyaluronic acid implantation showed that on the side treated with hyaluronic acid only minor tissue reaction was observed with a few weak adhesions. The polyethylene tubes were surrounded by a thin soft capsule, which was removed for histological examination. On the control side a more extensive tissue reaction was observed in all 5 cases, with a more marked adhesion formation. In the 3 animals which did not reject the tubes, a thick capsule was formed around the tubes. This was removed for histological examination. The microscopic picture of the capsules showed fewer cells and more fibrous tissue on the control side compared to the hyaluronic acid-treated side (Fig. 1 A and B).

Of the 35 rabbits submitted to tendon damage, 28 showed a significantly higher tensile strength of adhesions in the control leg where no hyaluronic acid was implanted; 3 rabbits showed a significantly higher tensile strength on the hyaluronic acid-treated side. Four of the animals showed no difference between the two sides; of these, 3 had weak adhesions on each side. In 5 of the rabbits, the adhesions on the control side were so strong that the tendon partly broke. In these cases strong adhesions were formed in the bottom of the tendon groove due to a periosteal reaction with new bone formation (Table 3).

DISCUSSION

The mechanism of the clinical improvement after intra-articular injections of hyaluronic acid is not clear. To restore the surface layer of hyaluronic acid is probably important, but there are other factors that may contribute to the improvement.

The biopolymer itself may provoke a reaction in the synovial tissue that has a controlling effect on the pathological process. When hyaluronic acid is injected into a normal or arthritic (degenerative or traumatic) joint, a rapid influx of mononuclear macrophage-like cells occurs (Fig. 2). These cells contain large (lysosomal) basophilic

TABLE 3. Formation of Adhesions Around Traumatized Tendons in Rabbits

Number Evaluated	*Less Reaction on HA Side*	*Less Reaction on Control Side*	*No Difference Observed Between HA and Control Side*
35	28	3	4

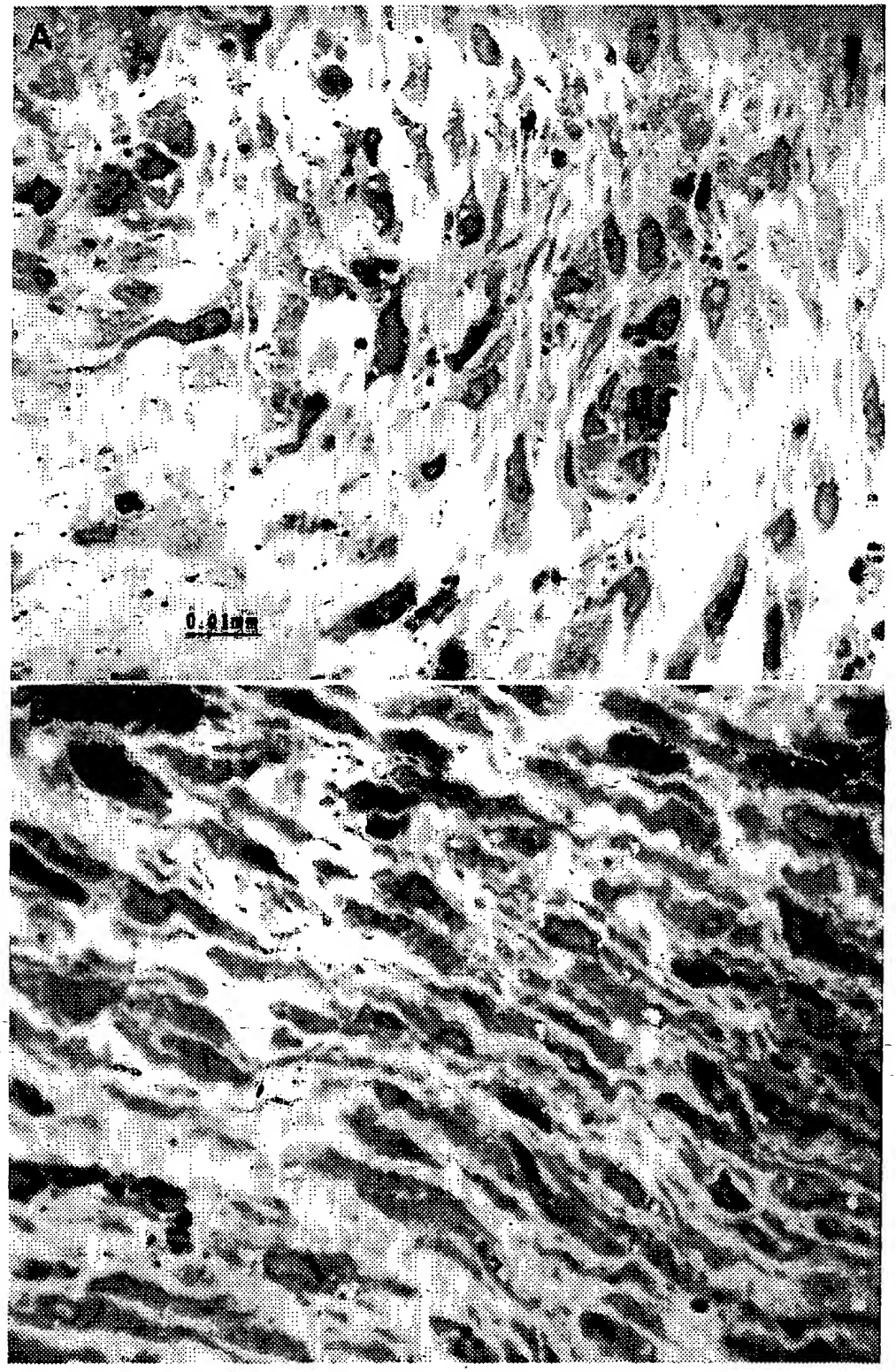
A
0.01µm

FIG. 1. Connective tissue capsule around polyethylene tube implanted with (A) and without (B) hyaluronic acid (H & E).

granules that give the acid phosphatase and periodic acid Schiff reactions. The amount of these cells in horse joint reaches peak value 24 hours after injection. At the same time, a considerable number of polymorphonuclear cells also are present in the joint; however, no clinical sign of inflammation is observed, such as swelling, pain, or increased skin temperature. It is possible that this macrophage invasion is instrumental in producing improvement of the condition of the joint. These cells are of phagocytic type, and they may participate in the removal of the injected hyaluronic acid. To ascertain that the presence of these cells is a response to the injection of hyaluronic acid itself and not to impurities, 40 ml of synovial fluid taken from several joints of a horse were centrifuged at 105,000 $\times$ g for 12 hours under sterile conditions. The hyaluronic acid concentration was approximately 5 times higher in the injected fluid than in the normal fluid, but the protein content was only slightly elevated. The highly viscous sediment, about 4 ml, was then injected into a previously unpunctured joint of the same horse from which the sample was collected. The cellular response was similar to that obtained after hyaluronic acid injection. This experiment suggests that the increase in large

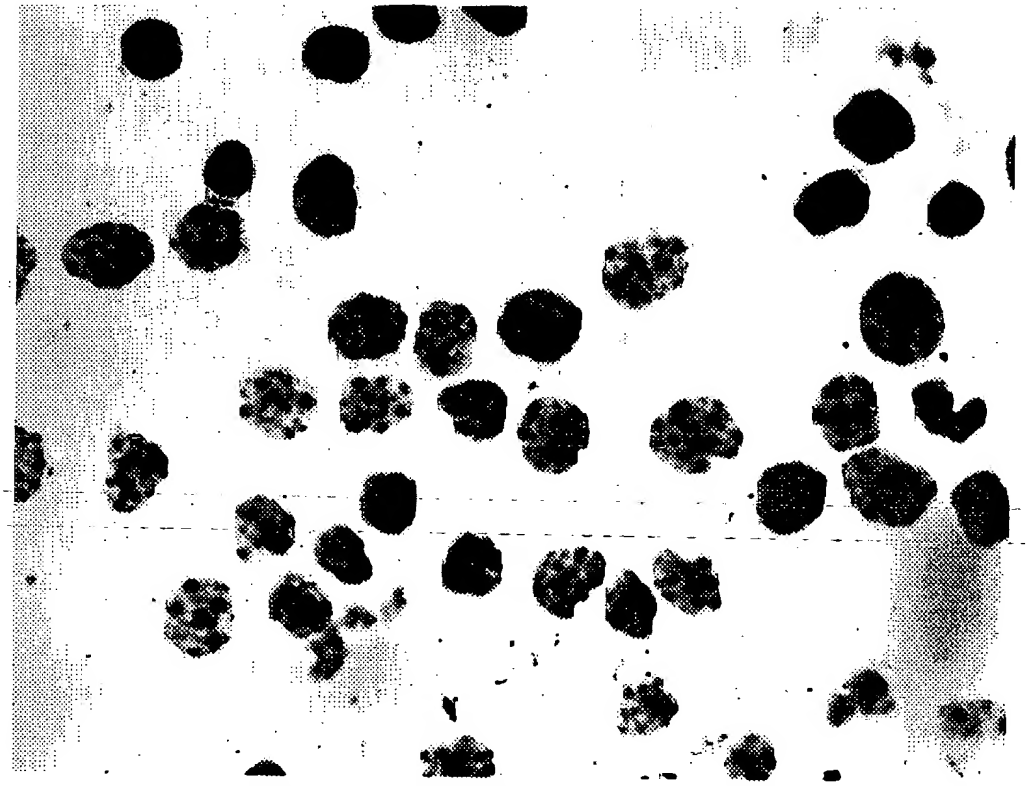

FIG. 2. Large mononuclear cells with lysosomal granules in the horse joint 24 hours after injection of 20 mg hyaluronic acid. Cells fixed in buffered glutaraldehyde were stained using Gomori's acid phosphatase method.

mononuclear cells represents a reaction to the increase in hyaluronic acid concentration. This concept is supported by the finding that the same hyaluronic acid preparations increase the macrophage content in the abdominal cavity of mice and rats (Balazs and Friberg, unpublished data).

SUMMARY

In dogs and owl monkeys, intra-articular implantation of hyaluronic acid (HA) in damaged joints significantly improved the healing process. Of 39 animals, 32 showed less connective tissue reaction with a smoother subcutaneous scar when the wound was treated with HA. In 5 animals tested, implanted foreign bodies showed less reaction if they were embedded in HA before the implantation. Twenty-eight out of 35 rabbits with tendon injuries had greatly reduced adhesion formation around the tendon after implantation of HA. In 12 race horses with traumatic arthritis, improvement was observed after intra-articular injections of HA mixed with Depo-Medrol, while 8 horses treated with Depo-Medrol alone did not improve to the same extent.

REFERENCES

1. Balazs, E. A.: Viscoelastic properties of hyaluronic acid and biological lubrication, Univ. Mich. Med. Cent. J. special issue, December 1968, pp. 255-259.
2. ———, Bloom, G. D., and Swann, D. A.: Fine structure and glycosaminoglycan content of the surface layer of articular cartilage, Fed. Proc. 25:1813-1816, 1966.
3. Balazs, E. A.: Some aspects of the aging and radiation sensitivity of the intercellular matrix with special regard to hyaluronic acid in synovial fluid and vitreous. *In* Engel, A. and Larsson, T., eds.: Thule International Symposium: Aging of Connective and Skeletal Tissue, Stockholm, Nordiska Bokhandelns Forlag, 1969; pp. 107-122.
4. ———, and Sweeney, D. B.: The injection of hyaluronic acid and reconstituted vitreous into the vitreous cavity. *In* McPherson, A., ed.: New and Controversial Aspects of Retinal Detachment, New York, Hoeber Medical Division, 1968; pp. 371-376.
5. Curri, S. B., and Campailla, E.: Acido jaluronico e condrogenesi: Influenza del mucoploysaccharide sui processi, riparativi della cartilagine, Biochim. Biol. Sper. VI:32, 1967.
6. Gibbs, D. A., Merrill, E. W., Smith, K. A., and Balazs, E. A.: Rheology of hyaluronic acid, Biopolymers 6:777-791, 1968.
7. Hunt, M. D. N.: Traumatic arthritis in young thoroughbreds, Proc. Roy. Soc. Med. 58:370-372, 1965.
8. Raker, C. W.: Clinical observations of bone and joint diseases in horses, Cornell Vet., LVIII: Supplement, January 1968.
9. Rydell, N. W., Butler, J., and Balazs, E. A.: Hyaluronic acid in synovial fluid. VI. Effect of intra-articular injection of hyaluronic acid on the clinical symptoms of arthritis in track horses, Acta Vet. Scand. 11:139-155, 1970.